Synthesis and Pharmacology of Benzoxazines as Highly Selective Antagonists at M₄ Muscarinic Receptors

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Previously, we reported on PD 102807 (**41**) as being the most selective synthetic M₄ muscarinic antagonist identified to date. Synthesized analogues of **41** showed no improvement in affinity and selectivity at that time. However, several newly synthesized compounds exhibit a 7-fold higher affinity at M₄ receptors and demonstrate a selectivity of at least 100-fold over all other muscarinic receptor subtypes. For example, compound **28** showed an affinity of $pK_i = 9.00$ at M₄ receptors and a selectivity of M₁/M₄ = 13 183-fold, M₂/M₄ = 339-fold, M₃/M₄ = 151-fold, and M₅/M₄ = 11 220-fold. This high selectivity along with high affinity has not been reported for any synthetic muscarinic antagonist, nor for natural occurring M₄ antagonists such as the M₄ selective Eastern Green Mamba venom MT3 (M₄ $pK_b = 8.7$, M₁/M₄ = 40-fold, M₂/M₄ ≥ 500-fold, M₃/M₄ ≥ 500-fold, and M₅/M₄ ≥ 500-fold). Derivative **24**, a compound with a high selectivity pattern as well, has been tested for in vivo efficacy. It was able to block the L-3,4-dihydroxyphenylalanine accumulation produced by CI-1017, an M₁/M₄ selective muscarinic agonist, in the mesolimbic region and striatum, which suggests that **24** is capable of crossing the blood−brain barrier and confirms the pharmacokinetic data obtained on this compound. This is evidence that suggests that agonist-induced increase in catecholamine synthesis observed in these regions is mediated by M₄ receptors.

Introduction

The five muscarinic receptor subtypes (M₁−M₅) belong to the superfamily of G-protein-coupled receptors and include seven hydrophobic regions, which form α-helical transmembrane (TM) domains connected by alternating intracellular and extracellular loops.[1] In most neuronal cells, M₁, M₃, and M₅ receptors are positively coupled to phospholipase C. This results in the formation of diacylglycerol, which subsequently activates protein kinase C, increases the release of inositol trisphosphate (IP₃), and then liberates free, intracellular Ca^{2+}. In contrast, M₂ and M₄ receptors are negatively coupled to adenylyl cyclase.[2] Because of their wide distribution in both peripheral and central tissues, muscarinic receptors appear to play a role in diseases such as glaucoma, bradycardia, asthma, psychosis, Alzheimer's disease, and Parkinson's disease.[3]

Muscarinic receptor subtypes also display a high degree of sequence identity among one another and differences in binding of agonists or antagonists are likely based on conformational dissimilarities rather than on single amino acid residues.[4] Nonselective ligands exhibit many undesirable side effects that limit their clinical usefulness. Newer synthesized compounds show some subtype selectivity, although none is so selective as to display a 100-fold greater affinity for one subtype over all others.[5] Thus, one goal is to identify and develop highly potent and selective molecules that overcome the limitations of presently available compounds.

Parkinsonism is caused by a loss of dopamine (DA) neurons, which causes an imbalance between the dopaminergic and the cholinergic system in the basal ganglia and results in tremor and movement dysfunction.[6] To decrease the cholinergic predominance, tremors can be treated with drugs such as trihexyphenidyl, an M₁/M₄ antagonist.[7] Data from rats show that M₄ muscarinic receptors are colocalized with DA D₁ receptors in the striatum.[8] The selective inhibition of M₄ receptors could result in enhanced DA D₁ receptor signaling, which in turn would lead to a reduced requirement for L-dopa, the standard therapy for Parkinsonism.[9] Another beneficial effect of anticholinergic treatment could also occur in the substantia nigra where muscarinic receptors directly influence the decarboxylation of L-dopa in the striatum.[10] On the basis of these findings, the M₄ subtype presents a potential target for Parkinson's research, and selective M₄ antagonists could show beneficial therapeutic effects without unwanted cholinergic side effects.

Compound **41**, a benzoxazine analogue, which exhibits high selectivity for the M₄ receptor and shows properties of a competitive antagonist, was recently reported.[11] The affinities and ratios found were as follows: pK_i/M₁ = 5.88, M₂ = 6.27, M₃ = 6.59, M₄ = 8.15, M₅ = 5.47, M₁/M₄ = 188-fold, M₂/M₄ = 77-fold, M₃/M₄ = 37-fold, and M₅/M₄ = 484-fold. Compound **41** was examined in whole cell functional assays measuring its ability to block agonist-induced changes in second messenger systems. In Hm1, Hm3, and Hm5 Chinese

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Scheme 1[a]

7a-e   **12a-s**   **26-40**
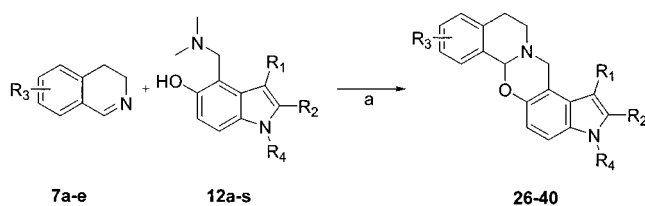
[a] Reagents: N_2, dioxane, Δ.

hamster ovarian (CHO) cells, **41** antagonized the ability of carbachol to activate phosphatidylinositol (PI) hydrolysis, while in Hm2 and Hm4 CHO cells it blocked the inhibition of forskolin-activated adenosine cyclic 3′,5′-phosphate (cAMP) accumulation produced by carbachol. These results confirmed the binding results and showed that **41** was an M_4 selective antagonist. Following these initial findings, additional analogues were synthesized, but none exhibited improved potency or selectivity.[11] In a continued search for a better antagonist, a second series of related compounds were prepared, of which some did show improved potency and selectivity.

This paper will report the affinity profiles for this new series of analogues on the five human receptor subtypes using transfected CHO cells with [3H]NMS (*N*-methylscopolamine) displacement binding.[12,13] Additionally, one of the more selective antagonists **24** was tested in vivo for the ability to block the stimulation of catecholamine synthesis induced by the M_1/M_4 agonist, CI-1017,[14] and for efficacy in a locomotor activity test (LMA).

Chemistry

The general synthesis of benzoxazines (**16**−**40**) is shown in Scheme 1. Substituted 3,4-dihydro-isoquinolines (**7a**−**e**) and Mannich base (**12a**−**s**) undergo a Diels−Alder reaction when heated in dioxane to yield benzoxazines **16**−**40**. The synthesis of the starting materials is described in Schemes 2−3 and 5−6.

Scheme 2 depicts the synthesis of 2-(3-methylsulfanyl-phenyl)ethylamine (**5a**),[15] which is the starting material for the 6-methylsulfanyl-3,4-dihydro-isoquinoline derivative **7a**. (3-Methylsulfanyl-phenyl)methanol (**1**) is converted to 1-chloromethyl-3-methylsulfanyl-benzene (**2**) using thionyl chloride. The cyanide compound (3-methylsulfanyl-phenyl)acetonitrile (**3**) is synthesized by treating **2** with potassium cyanide in the presence of 18-crown-6 ether. Standard reduction conditions (e.g.,
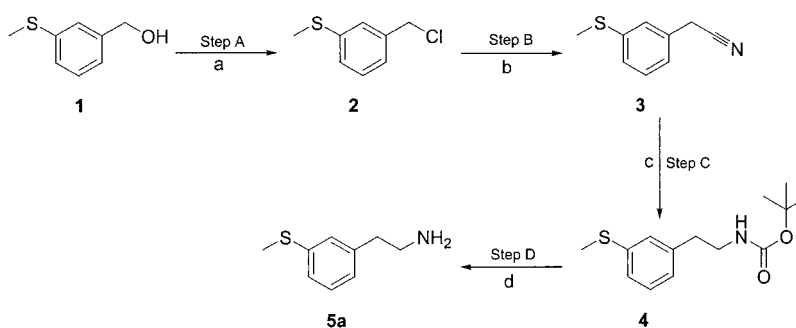
Raney nickel) in the presence of BOC anhydride gives **4**. Deprotection of **4** with 2,2,2-trifluoro-acetic acid yields **5a**.

Scheme 3 illustrates the synthesis of 3,4-dihydro-isoquinolines (**7a**−**e**) of which **5b**,**c** are commercially available. Condensation of 3-(substituted-phenyl)ethylamines (**5a**−**c**) with formic acid gives *N*-phenethyl-formamides (**6a**−**c**). A Bischler−Napieralski ring closure yields **7a**−**d**. Treatment of 6-methoxy-3,4-dihydro-isoquinoline (**7d**) with HBr (47%) gave 3,4-dihydro-isoquinoline-6-ol (**7e**).

Scheme 4 illustrates the synthesis of 1-(3-methyl-[1,2,4]oxadiazol-5-yl)propan-2-one (**9a**), the starting material for the preparation of the 5-hydroxy-indole **12a** in Scheme 5. Treatment of *N*-hydroxy-acetamidine hydrochloride (**13**) with 2,2,6-trimethyl[1,3]dioxin-4-one in the presence of triethylamine gave 1-(3-methyl[1,2,4]-oxadiazol-5-yl)propan-2-one (**9a**).

Scheme 5 depicts the preparation of the Mannich bases **12a**−**p**. Condensation of **9a**−**j** and ammonia or a primary amine yielded the enamino compounds **10a**−**p**, which were converted to substituted 5-hydroxy-indoles (**11a**−**p**) using the Nenitzescu reaction. Standard Mannich conditions afforded Mannich bases (**12a**−**p**).
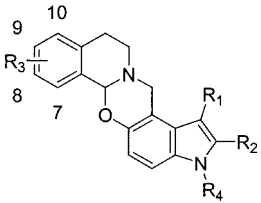
Scheme 6 illustrates the synthesis of Mannich bases **12q**−**s**. Treatment of 5-hydroxy-2-methyl-1*H*-indole-3-carboxylic acid benzyl ester (**11d**) with TIPS-chloride gave 2-methyl-5-triisopropylsilanyloxy-1*H*-indole-3-carboxylic acid benzyl ester (**13**). Reductive debenzylation with Pd/C/hydrogen yielded 2-methyl-5-triisopropylsi-lanyloxy-1*H*-indole-3-carboxylic acid (**14**). Activation of **14** with PyBOP and treatment with ethyl-methylamine gave 5-hydroxy-2-methyl-1*H*-indole-3-carboxylic acid ethyl-methyl-amide (**11r**). 5-Hydroxy-2-methyl-1*H*-in-dole-3-carboxylic acid ethyl ester (**11q**) was treated with 20% aqueous HCl to yield 2-methyl-1*H*-indole-5-ol (**15**). Alkylation of **15** with benzylbromide yielded 3-benzyl-2-methyl-1*H*-indol-5-ol (**11s**). Compounds **11q**−**s** were treated with formaldehyde and dimethylamine to give the Mannich bases **12q**−**s**.

Results

Several synthesized benzoxazine analogues of general formula shown in Scheme 1 (**16**−**40**) have been synthesized and characterized as M_4 selective muscarinic antagonists (Table 1).

This study focuses on different substitutions at positions 1−3 of the indole moiety and on substituents of

Scheme 2[a]

1   **2**   **3**

5a   **4**

[a] Reagents: (a) $SOCl_2$, benzene, room temperature; (b) KCN, 18-crown-6, CH_3CN, room temperature; (c) Ra/Ni, BOC anhydrous, MeOH, room temperature; (d) CF_3COOH, CH_2Cl_2, room temperature.

Table 1. Binding Results of Benzoxazines

example	R¹	R²	R³	R⁴	M₁	M₂	M₃	M₄	M₅	formula[c]	mp (°C)
					$pK_i{}^a$		

example	R^1	R^2	R^3	R^4	M_1	M_2	M_3	M_4	M_5	formula[c]	mp (°C)
	CO_2CH_3	CH_3	9-OMe	CH_3	5.93	6.38	6.40	6.72	5.44	$C_{23}H_{24}N_2O_4$	194–195
	$CO_2CH_2CH=CH_2$	CH_3	9-OMe	H	5.88	5.97	6.39	7.82	5.65	$C_{24}H_{24}N_2O_4 \cdot 0.1H_2O$	188–190
	$CO_2CH_2CH=CH_2$	CH_3	9-OMe	(p-OMe)Bz	6.70	6.62	6.17	7.14	6.30	$C_{32}H_{32}N_2O_5 \cdot 0.04H_2O$	175–176
	$CO_2CH_2C_6H_5$	CH_3	9-OMe	H	5.93	5.87	5.86	7.14	5.95	$C_{28}H_{26}N_2O_4$	176–178
	$CO_2(CH2)_3CH_3$	CH_3	9-OMe	H	5.88	5.63	5.99	7.17	5.60	$C_{25}H_{28}N_2O_4$	177–178
	$CO_2CH_2CH_3$	CH_2CH_3	9-OMe	H	5.66	5.87	7.04	8.40	5.51	$C_{24}H_{26}N_2O_4$	183–184
	$CO_2CH_2CH_3$	CH_2CH_3	9-OMe	benzyl	6.44	6.43	7.30	7.01	6.32	$C_{31}H_{32}N_2O_4 \cdot 0.04H_2O$	75–85
	$CO_2CH_2CH_3$	CH_2CH_3	9-OMe	CH_3	5.82	6.15	6.66	8.52	5.51	$C_{25}H_{28}N_2O_4 \cdot 0.06H_2O$	172–174
	$CO_2CH_2CH_3$	**$(CH_2)_2CH_3$**	**9-OMe**	**H**	**5.69**	**5.97**	**7.15**	**8.70**	**5.65**	**$C_{25}H_{28}N_2O_4 \cdot 0.11H_2O$**	**194–196**
	$CO_2CH_2CH_3$	$(CH_2)_2CH_3$	9-SMe	H	6.06	5.84	7.00	9.00	5.62	$C_{25}H_{28}N_2O_3S \cdot 0.02H_2O$	185–186
	$CO_2CH_2CH_3$	$(CH_2)_2CH_3$	9-SMe	CH_3	5.48	5.42	6.64	8.70	5.77	$C_{26}H_{30}N_2O_3S \cdot 0.27H_2O$	139–143
	$CO_2CH_2CH_3$	$(CH_2)_2CH_3$	9-OMe	CH_3	5.88	6.29	7.15	8.70	5.47	$C_{26}H_{30}N_2O_4$	150–151
	$CO_2CH_2CH_3$	**$(CH_2)_3CH_3$**	**9-OMe**	**H**	**4.88**	**6.47**	**6.82**	**9.00**	**4.95**	**$C_{26}H_{30}N_2O_4$**	**180–182**
	$CO_2CH_2CH_3$	$(CH_2)_3CH_3$	9-SMe	H	6.06	5.66	6.47	8.52	5.83	$C_{26}H_{30}N_2O_3S$	159–161
	$CO_2CH_2CH_3$	$(CH_2)_4CH_3$	9-OMe	H	4.69	5.14	6.34	7.96	5.27	$C_{27}H_{32}N_2O_4$	170–172
	$CO_2CH_2CH_3$	$(CH_2)_5CH_3$	9-OMe	H	5.51	5.34	6.55	7.85	>4.55	$C_{28}H_{34}N_2O_4$	157–160
	$CO_2CH_2CH_3$	CH_3	9-Cl	H	5.29	5.49	5.56	6.44	5.27	$C_{22}H_{21}ClN_2O_3 \cdot 0.7H_2O$	196–197
	$CO_2CH_2CH_3$	CH_3	7-Cl	H	>5.36	5.61	5.29	5.71	5.32	$C_{22}H_{21}ClN_2O_3$[d]	188–189
	$CO_2CH_2CH_3$	CH_3	9-SMe	H	5.75	5.66	6.64	8.15	5.71	$C_{23}H_{24}N_2O_3S \cdot 0.02H_2O$	188–189
	$CO_2CH_2CH_3$	CH_3	9-OH	H	>4.66	>4.57	4.59	>4.91	>4.55	$C_{22}H_{22}N_2O_4$[e]	241–243
	$CO_2CH_2CH_3$	CH_3	9-OMe	CH_2CH_3	5.88	6.66	6.58	6.96	5.60	$C_{25}H_{28}N_2O_4$[f]	173–175
	$CO_2CH_2CH_3$	CH_3	9-OMe	CH_3	5.70	6.29	6.04	7.82	5.43	$C_{24}H_{26}N_2O_4 \cdot 0.1H_2O$	174–176
	$CONCH_3C_2H_5$	CH_3	9-OMe	H	4.66	5.39	4.81	5.01	4.71	$C_{24}H_{27}N_3O_3$[g]	162–164
	$CH_2C_5H_6$	CH_3	9-OMe	H	5.60	5.27	5.69	5.87	5.95	$C_{27}H_{26}N_2O_2 \cdot 0.04H_2O$	196–198
	oxadiazole[b]	CH_3	9-OMe	H	5.12	5.66	5.51	7.21	5.21	$C_{23}H_{22}N_4O_3 \cdot 0.19H_2O$	195–197
(PD102807)	$CO_2CH_2CH_3$	CH_3	9-OMe	H	5.88	6.27	6.59	8.15	5.47	$C_{23}H_{24}N_2O_4$	190–192

[a] The affinity of these compounds for the five human receptor subtypes (M_1–M_5) was determined by [³H]NMS binding using membranes from transfected CHO cells. All compounds were tested at least three times; the value shown is given as the geometric mean. The standard error of the mean (SEM) for all compounds tested ranged from 6 to 18%. Complete protocol is described by Dörje et al.[12] and Buckley et al.[13] The K_i values of the test compounds were derived from IC_{50} values using the Cheng–Prusoff equation, $K_i = IC_{50}/(1 + L/K_d)$, with the radioligand concentration L = 0.1 nM and the following equilibrium dissociation constants K_D of [³H]NMS, determined in previous saturation binding experiments (nM): M_1 (1.28), M_2 (0.85), M_3 (0.94), M_4 (3.10), and M_5 (0.79).[30] [b] Oxadiazole = 3-methyl[1,2,4]oxadiazol-5-yl. [c] All compounds have analytical results within ±0.4% of theoretical values unless otherwise noted. Some difficulty was found in obtaining combustion analysis in the indicated compounds due to the propensity of these compounds to retain solvents. [d] High mass: calcd, 397.1319; found, 397.1325. [e] High mass: calcd, 379.1658; found, 379.1651. [f] High mass: calcd, 421.2127; found, 421.2122. [g] High mass: calcd, 406.2130; found, 406.2137.
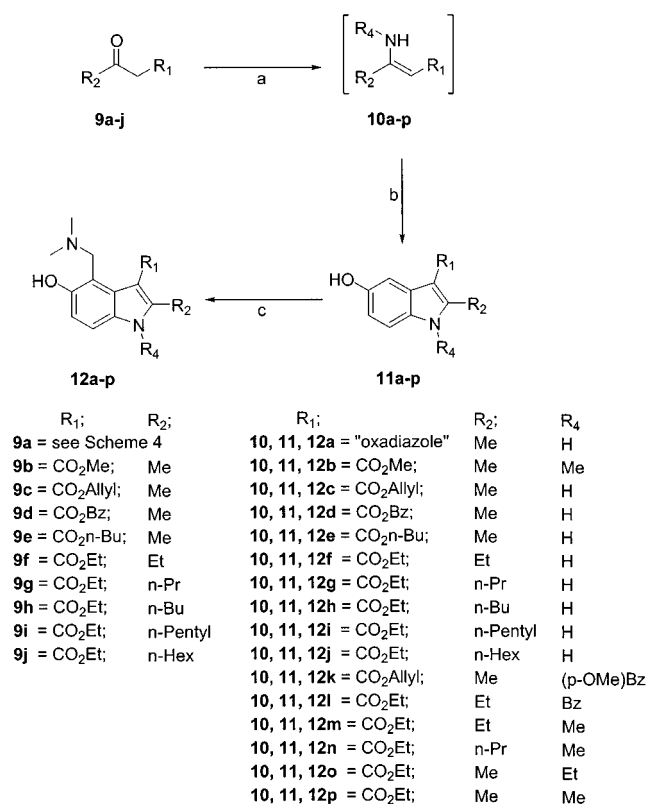
Scheme 3[a]

5a = SCH₃	**6a** = SCH₃
5b = Cl	**6b** = Cl
5c = OCH₃	**6c** = OCH₃

7a = 6-SCH₃
7b = 6-Cl
7c = 8-Cl
7d = 6-OCH₃
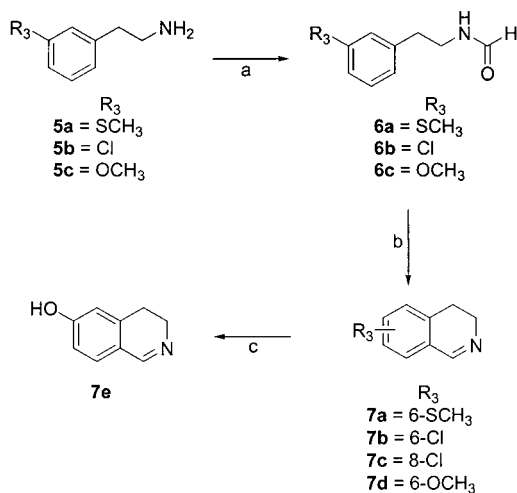
[a] Reagents: (a) HCOOH, Δ; (b) POCl₃, benzene, Δ; (c) HBr (47%), Δ.

Scheme 4[a]
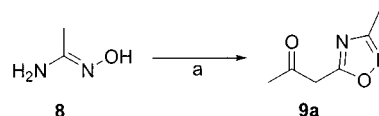
[a] Reagents: (a) 2,2,6-Trimethyl[1,3]dioxin-4-one, (Et)₃N, Δ.

the aromatic part of the tetrahydro isoquinoline moiety. Initial analogues included various ester groups other than ethyl ester at position 3 of the indole structure (R^1). These analogues led to lower affinity at M_4 receptors (**16**, **17**, **19**, and **20**). However, when R^1 was either an amide moiety (**38**) or a benzyl group (**39**), a striking decrease of affinity of 351–1396-fold vs **41** was observed. In contrast, an ester bioisostere, [1,2,4]oxadiazole (**40**), demonstrated only a 7-fold decrease in affinity while maintaining the selectivity (M_1/M_4 = 58-fold, M_2/M_4 = 71-fold, M_3/M_4 = 41-fold, and M_5/M_4 = 89-fold) as compared to **41**.

Analogues with longer alkyl chains (**21**, **23–31**) at position 2 of the indole part (R^2) exhibited increased

Scheme 5[a]

R1;	R2;
9a = see Scheme 4	
9b = CO_2Me;	Me
9c = CO_2Allyl;	Me
9d = CO_2Bz;	Me
9e = CO_2n-Bu;	Me
9f = CO_2Et;	Et
9g = CO_2Et;	n-Pr
9h = CO_2Et;	n-Bu
9i = CO_2Et;	n-Pentyl
9j = CO_2Et;	n-Hex

	R1;	R2;	R4
10, 11, 12a = "oxadiazole"		Me	H
10, 11, 12b = CO_2Me;		Me	Me
10, 11, 12c = CO_2Allyl;		Me	H
10, 11, 12d = CO_2Bz;		Me	H
10, 11, 12e = CO_2n-Bu;		Me	H
10, 11, 12f = CO_2Et;		Et	H
10, 11, 12g = CO_2Et;		n-Pr	H
10, 11, 12h = CO_2Et;		n-Bu	H
10, 11, 12i = CO_2Et;		n-Pentyl	H
10, 11, 12j = CO_2Et;		n-Hex	H
10, 11, 12k = CO_2Allyl;		Me	(p-OMe)Bz
10, 11, 12l = CO_2Et;		Et	Bz
10, 11, 12m = CO_2Et;		Et	Me
10, 11, 12n = CO_2Et;		n-Pr	Me
10, 11, 12o = CO_2Et;		Me	Et
10, 11, 12p = CO_2Et;		Me	Me

[a] Reagents: (a) R_4-NH_2, room temperature; (b) 1,4-benzoquinone, CH_3COOH, room temperature; (c) HCHO, $HN(CH_3)_2$, ethanol, 50 °C.

affinity culminating from propyl to pentyl (**24**−**30**). The affinity dropped significantly with compound **31**, which displayed a hexyl chain at R^2. Compound **28**, where R^2 equals butyl, exhibited the greatest selectivity (M_1/M_4 = 13 183-fold, M_2/M_4 = 339-fold, M_3/M_4 = 151-fold, and M_5/M_4 = 11 220-fold).

In further efforts to develop a structure−activity relationship, the alkylation of the indole moiety was investigated. Methylation of the nitrogen of the indole moiety (R^4) yielded compounds with slightly lower affinity at M_4 receptors (**23**, **26**, **27**, and **37**). An ethyl group (**36**) at R^4 or even more bulky groups such as benzyl (**22**) or 4-methoxybenzyl (**3**) lead to unselective compounds with decreased affinity.

Benzoxazines with a substituent other than methoxy at the isoquinoline moiety were synthesized in order to examine any potential influence in binding. Compounds with a methylthioether at position 9 (**25**, **26**, **29**, and **34**) bound either equally or slightly stronger than compounds with a methoxy group (**24**, **27**, **28**, and **44**) at R^3. Derivatives with a 7-chloro (**33**) or 9-hydroxy group (**35**) at R^3 demonstrated a much lower affinity at M_4 receptors (285−1758-fold) and almost no selectivity for the M_4 receptor in comparison to **41**. In addition, the 9-chloro compound (**32**) elicited a decreased affinity as compared with the parent compound (53-fold).

Neurochemistry. CI-1017 at 10 mg/kg, ip significantly increased catecholamine synthesis (as assessed by elevation in levels of L-3,4-dihydroxyphenylalanine (DOPA)) in the DA-enriched striatum and mesolimbic regions of rat brain (Figure 1).[14] Moreover, compound **24** administered at 30 mg/kg, ip significantly blocked the increase in DA synthesis induced by CI-1017. The compound by itself did not alter DA synthesis.

Bioavailability. Compound **24** has been tested for brain penetration, bioavailability, and clearance. The compound penetrates into the brain but has low bioavailability (<5%) and high clearance (Tables 2 and 3).

Locomotive Activity. Compound **24** significantly decreased the locomotive activity of rats in the dark 50 and 70% when given orally at doses of 30 and 100 mg/kg, respectively (Table 4).

Discussion

Although it is more than a decade ago that the five distinct muscarinic receptors were identified, a potent and highly selective (>100-fold) synthetic muscarinic receptor agonist or antagonist has not been reported.[16] Initially, **24** was reported to be more selective than **41** and biological tests were carried out with this substance. Subsequently, **28** showed an even higher selectivity and affinity at M_4 muscarinic receptors than **24**. Some closely related compounds of **28** (Table 1: **25**, **26**, and **29**) also belong to the first muscarinic antagonists that provide the opportunity to antagonize the M_4 subtype with more than 100-fold selectivity over all other muscarinic receptor subtypes (M_1−M_3, M_5). The Eastern Green Mamba toxin MT3, the previously most selective M_4 antagonist known, is not as selective as **28** at M_1 receptors (40-fold).[17] Additionally, compound **24** is ca-
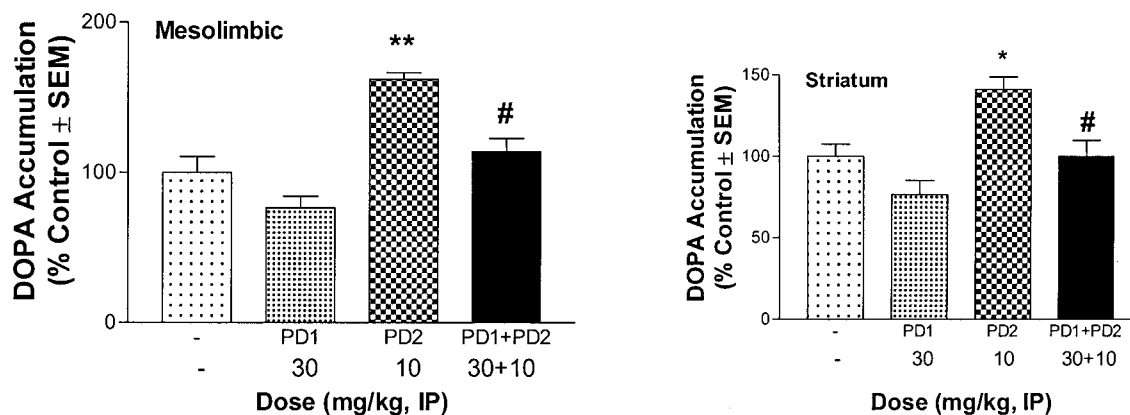
Figure 1. PD1 (**24**) and PD2 (C1-1017) were dosed 25 and 5 min before NSD 1015 (100 mg/kg, IP). Rats were sacrificed 30 min after NSD 1015 was administered. Each value is a mean of four animals and is expressed as percent of controls that were 740 ± 78 and 1211 ± 91 ng/g for mesolimbic and striatum, respectively. **$P < 0.001$; *$P < 0.05$ vs control group; and #$P < 0.01$ vs PD2-treated group.

Scheme 6[a]

R₁ legend:

12q = CO_2Bz
12r = CO_2Et
12s = $CON(Et)Me$
12t = Bz
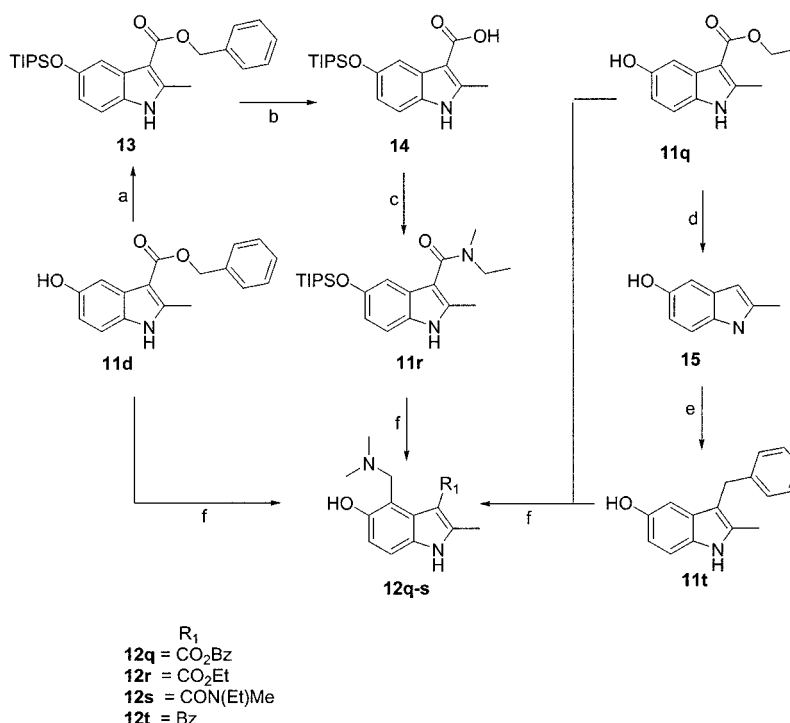
[a] Reagents: (a) Imidazole, TIPS-Cl, DMF, room temperature; (b) Pd/C, H₂, HCOOH, ethanol, room temperature; (c) PyBOP, HOBT, N(n-C₃H₇)₃, DMF, room temperature; (d) aqueous HCl (20%), Δ; (e) benzylbromide, K₂CO₃, acetonitrile, Δ; (f) HCHO, HN(CH₃)₂, ethanol, 50 °C.

Table 2. Determination of Bioavalability of Compound **24** in Male Wistar Rats

parameter	units	mean of 3 rats	SD	% RSD	mean of 3 rats	SD	% RSD
route		IV[c]			PO[d]		
dose	ng/kg	1 000 000			5 000 000		
AUC extrap[a]	ng × hours/mL	282.334	39.645	14.0			
AUC (0-tldc)[b]	ng × hours/mL				61.37	20.969	34.2
clearance	mL/min/kg	59.777	7.971	13.3			

[a] AUC extrap = area under the curve extrapolated. [b] AUC (0-tldc) = area under the curve from zero time to time of last detectable concentration. [c] IV = intraveneous. [d] PO = per os.

Table 3. Plasma and Brain Concentrations of **24** Following a 1 h Infusion of 1 mg/kg **24** to Rats

	brain wt (g)	brain (ng/g)	plasma (ng/mL)	brain/ plasma
mean of 3 rats	0.923	311	104	3.2
SD[a]	0.126	50.1	28	1.1
% RSD[b]	13.6	16.1	26.9	34.3

[a] SD = standard deviation. [b] % RSD = % relative standard deviation.

pable of crossing the blood—brain barrier, which makes it a very interesting tool to investigate the function of the M₄ receptor in the central nervous system. For example, the selective blockade of M₄ receptors could reveal their potential role in Parkinson's disease.

It is difficult to determine why selected compounds bind better and show more selectivity than others within a chemical series. However, the molecular structure of compound **24** and analogues may provide a unique binding pattern. The hemiaminal moiety found in these compounds is known for its chemical instability. It
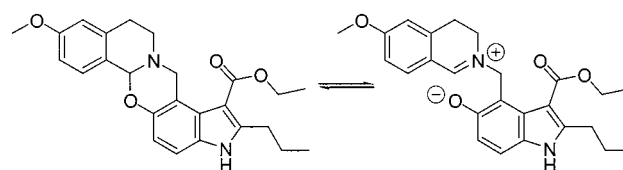
Figure 2. Structure of proposed ring opening.

might be possible that the hemiaminal structure of compound **24** opens up in the binding pocket of M₄ receptors, providing an imminium cation, which may be able to form an ionic bond with the receptor protein (Figure 2). The corresponding phenolate anion could then interact with a hydrogen donor. The ring opening may allow the molecule to twist around the methylene bridge between indole and isoquinoline moieties. Certain amino acid residues in M₄ receptors may be have the "right" distance to these charges. These interactions could then allow the compounds to exhibit high affinity and selectivity to the M₄ receptor. Because of conformational differences in the other receptor subtypes

Table 4. Total Distance Travelled (cm) after 30 Minute Acclimation in Chambers (Locomotor Activity)

vehicle PO[a]	SEM	**24**: 10 mg/kg PO[a]	SEM[b]	**24**: 30 mg/kg PO[a]	SEM	**24**: 100 mg/kg PO[a]	SEM
3440.67	459.84	2908.83	315.98	1583.82	307.00	836.50	332.25

[a] PO = per os. [b] SEM = standard error of measurement.

(M_1–M_3, M_5), this tight bonding might not be possible, which subsequently leads to lower affinities of more than 100-fold. There is evidence in the literature that hydrogen-donating residues in the binding pocket, such as threonin and tyrosin, could form H-bonds with a ligand.[18] To show that compound **24** opens to the imminium cation in solution, the compound was dissolved in methanol and treated with sodium borohydride at room temperature. The reaction resulted in a hydrogenated phenol derivative. These sorts of reduced compounds were identified as byproducts in the previous study and were tested for activity toward the five muscarinic receptor subtypes. All reduced compounds tested demonstrated an affinity below 10 μM. In addition, a methylated analogue of **41** at the basic nitrogen forming a quaternary ammonium salt demonstrated no affinity at the muscarinic receptors.[11] The methylation prevents the oxazine ring from opening.

To show in vivo efficacy and to take advantage of the high selectivity of compound **24**, DOPA accumulation in rats was examined. As previously reported for xanomeline,[19] the M_1/M_4 agonist CI-1017 produced an increase in DA synthesis in the DA-enriched striatum and mesolimbic regions that is indicative of an increase in DA turnover.[20] Evidence exists that acetylcholine enhances DA release by agonist actions on dopaminergic terminals both in vitro and in vivo and that nonselective muscarinic antagonists block this effect.[21–23] This is further confirmed in the present study as the selective M_4 antagonist **24** blocked the increase in DA synthesis induced by CI-1017. Although the muscarinic receptor(s) that modulates DA synthesis is not rigidly defined, there is evidence that it has properties consistent with the M_1 subtype.[24,25] However, mRNA for both M_1 and M_4 receptors as well as M_1 and M_4 receptor protein has been documented in these regions.[8,26,27] The neurochemical data showing that the selective M_4 antagonist **24** totally blocked the effects of CI-1017, which has activity at both M_1 and M_4 receptors, suggest that the increase in catecholamine synthesis induced in the striatum and mesolimbic region may be mediated mainly by M_4 receptors.

Conclusion

In summary, compound **28** and some of the newly synthesized compounds show the highest selectivity known to date for the M_4 muscarinic receptor subtype. These compounds can be used as a tool for pharmacological studies for the unambiguous identification of the M_4 muscarinic receptor subtype. The in vivo efficacy of **24** could be shown in both the LMA and the neurochemical test (DOPA accumulation). Patients with movement disorders such as Parkinson's disease might benefit from a treatment with a highly selective M_4 muscarinic receptor antagonist, avoiding common side effects such as cognitive dull known from other unselective antagonists.

Experimental Section

Materials and Methods. High-resolution mass spectra were recorded using 4′,5′-dinitrofluorescein as standard. Melting points are uncorrected. [1]H nuclear magnetic resonance (NMR) spectra were obtained on a 400 MHz unit. Phenolic protons of compounds **7e**, **12g**, and the protons of the amine of **5a** do not give a signal, probably due to a deuterium exchange with the solvent. A 70–230 mesh silica gel was used in medium-pressure liquid chromatography (MPLC). Tetrahydrofuran (THF) was dried by distillation from Na°/9-fluorene. All other reagents were used as received.

Preparation of 9-Methoxy-2-propyl-11,12-dihydro-3*H*,-6a*H*,13*H*-6-oxa-3,12a-diaza-benzo[a]cyclopent[h]anthracene-1-carboxylic Acid Ethyl Ester (24) (Scheme 1). Compounds **12g** (1.2 g, 3.94 mmol) and **7d** (0.64 g, 3.94 mmol) were refluxed in dioxane (20 mL) for 4 h under a stream of nitrogen. The solution was concentrated, acetone (2 mL) was added, and the solution was allowed to stand for approximately 16 h at 5 °C. The white precipitate was filtered and dried in a vacuum oven at room temperature to give the desired product (**24**) (0.5 g, 30%). ^{1}H NMR (DMSO-d_6): δ = 0.88 (t, J = 7.3 Hz, 3H), 1.28 (t, J = 7.1 Hz, 3H), 1.62–1.65 (m, 2H), 2.71–2.76 (m, 2H), 2.86–2.94 (m, 3H), 2.99–3.04 (m, 1H), 3.74 (s, 3H), 4.13–4.23 (m, 3H), 4.67 (d, ^2J = 17.5 Hz, 1H), 5.61 (s, 2H), 6.51 (d, J = 8.5 Hz, 1H), 6.71 (s, 1H), 6.77 (d, J = 8.5 Hz, 1H), 7.06 (d, J = 8.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 1H). MS: m/z 421.2 (M + 1)$^+$; mp 194–196 °C. Anal. ($C_{25}H_{28}N_2O_4$) C, H, N. The compounds **25**–**40** and **16**–**23** were obtained following the preparation similar to that of **24**. The isomers **32** and **33** were separated by chromatography on silica gel eluting with CH_2Cl_2:MeOH 98:2.

Preparation of 1-Chloromethyl-3-methylsulfanyl-benzene (2) (Scheme 2). To a solution of **1** (23.7 g, 154 mmol) in dry benzene (150 mL),[15] thionyl chloride (27.4 g, 230 mmol) was slowly added with cooling (5 °C). The ice bath was removed, and the reaction mixture was stirred for 48 h at room temperature. The solution was concentrated to yield a dark residue, which was used without further work up.

Preparation of (3-Methylsulfanyl-phenyl)acetonitrile (3) (Scheme 2). Potassium cyanide (10.3 g, 158 mmol) was added to a solution of **2** (26.7 g, 155 mmol) and 18-crown-6 ether (2.6 g, 10 mmol) in dry acetonitrile and was stirred for 48 h at room temperature. Dichloromethane (400 mL) was then added to the reaction mixture upon which a white precipitate was formed. The suspension was filtered, and the filtrate was washed with water (2 × 150 mL), dried (Na_2SO_4), concentrated in vacuo, and distilled under reduced pressure (<1 mmHg, 110 °C) to give a colorless oil (**3**) (22.9 g, 91%). ^{1}H NMR (DMSO-d_6): δ = 2.44 (s, 3H), 3.98 (s, 2H), 7.07–7.09 (m, 1H), 7.17–7.19 (m, 2H), 7.28–7.32 (m, 1H). MS: m/z 161.9 (M − 1)$^-$.

Preparation of (3-Methylsulfanyl-phenyl)ethyl]carbamic Acid *tert*-Butyl Ester (4) (Scheme 2). Raney nickel (18 g) was added to a solution of **3** (22.9 g, 0.14 mol) and *tert*-butoxycarbonyl anhydride (BOC anh., 47 g, 0.25 mol) in methanol (200 mL) and shaken for 48 h at room temperature. The mixture was filtered and concentrated in vacuo. Purification via MPLC using hexane/ethyl acetate gradient from 5 to 20% ethyl acetate gave the desired product (**4**) (12.0 g, 33%). ^{1}H NMR (DMSO-d_6): δ = 1.32 (s, 9H), 2.46 (s, 3H), 2.60–2.64 (m, 2H), 3.06–3.11 (m, 2H), 6.84 (m, 1H), 6.91–6.92 (m, 1H), 7.02–7.05 (m, 2H), 7.16–7.20 (m, 1H). MS: m/z 268.0 (M + 1)$^+$.

Preparation of 2-(3-Methylsulfanyl-phenyl)ethylamine (5a) (Scheme 2). To a solution of **4** (12 g, 45 mmol) in CH_2Cl_2 (60 mL) was added trifluoroacetic acid (40 mL) and stirred for 10 min at room temperature. After the reaction was over, as visually indicated by the cessation of the evolution of gases (15 min), sodium hydroxide was added portionwise to pH > 13. The emulsion was extracted with CH_2Cl_2 (3 × 100 mL), dried (MgSO$_4$), and concentrated in vacuo to give an orange oil (**5a**) (7.4 g, 99%). ^{1}H NMR (DMSO-d_6): δ = 2.44 (s, 3H), 2.59–2.63 (m, 2H), 3.04–3.09 (m, 2H), 6.90–6.92 (m, 1H), 7.01–7.04 (m, 2H), 7.14–7.19 (m, 1H). MS: m/z 167.9 (M + 1)$^+$.

Preparation of *N*-[2-(3-Methoxy-phenyl)ethyl]formamide (6c) (Scheme 3). A solution of **5c** (50.0 g, 0.33 mol) in formic acid (60 mL) was refluxed for approximately 16 h. Water (250 mL) was added to the reaction mixture, and the emulsion was extracted with ethyl acetate (2 × 150 mL). The organic layer was separated, dried (Na_2SO_4), and concentrated in vacuo

to give the desired product **6c** (43.1 g, 73%). ^{1}H NMR (DMSO-d_6): $\delta = 2.65$ (t, $J = 7.1$ Hz, 2H), 3.25−3.30 (m, 2H), 3.69 (s, 3H), 6.72−6.74 (m, 3H), 7.16 (t, $J = 6.8$ Hz, 1H), 7.94 (s, 1H), 8.01 (bs, 1H). MS: m/z 180.1 (M + 1)$^+$. The compound **6b** was obtained following the preparation similar to that of **6c**.

Preparation of 6-Methoxy-3,4-dihydro-isoquinoline (7d) (Scheme 3). Phosphorus oxychloride (80 mL, 0.85 mol) was added dropwise to **6c** (41.1 g, 0.24 mol), and the mixture was refluxed for 1 h. The reaction mixture was then cooled to room temperature, and hexane was added and decanted off (3 × 100 mL). Water (200 mL) was added slowly to the dark oily solution with stirring. The mixture was basified with NaOH to pH > 13, extracted with ethyl acetate, dried (Na$_2$SO$_4$), and concentrated in vacuo to afford the desired product **7d** (26.1 g, 67%). ^{1}H NMR (DMSO-d_6): $\delta = 2.61$ (t, $J = 7.7$ Hz, 2H), 3.53 (t, $J = 7.7$ Hz, 2H), 3.74 (s, 3H), 6.76 (s, 1H), 6.81 (d, $J = 8.3$ Hz, 1H), 7.27 (d, $J = 8.3$ Hz, 1H), 8.18 (s, 1H). MS: m/z 162.1 (M + 1)$^+$. The compound **7a** was obtained following the preparation similar to that of **7d**.

Preparation of 6-Chloro-3,4-dihydro-isoquinoline (7b) (Scheme 3). Polyphosphoric acid (50 g) was added to **6b** (3.7 g, 0.02 mol), and the mixture was heated for 20 min at 140 °C. The reaction mixture was then poured on ice chips (30 g). Water (100 mL) was added to the dark oily solution with stirring. The mixture was basified with NaOH to pH > 13, extracted with ethyl acetate, dried (Na$_2$SO$_4$), and concentrated in vacuo to afford the desired products **7b,c** as a mixture (1.1 g, 33%). MS: m/z 165.1 (M + 1)$^+$ and 167.1 (M + 1)$^+$. The mixture was used without further workup for the synthesis of **17** and **18**.

Preparation of 3,4-Dihydro-isoquinolin-6-ol HBr (7e) (Scheme 3). Compound **7d** (2.5 g, 15.4 mmol) was refluxed in 47% HBr (10 mL) for 3 h under nitrogen. The solution was concentrated in vacuo. The residue was recrystallized from ethanol/ethyl acetate (1:1) to yield purple crystals of 3,4-dihydro-isoquinolin-6-ol-HBr (**7e**) (1 g, 44%). ^{1}H NMR (DMSO-d_6): $\delta = 3.02$ (t, $J = 8.1$ Hz, 2H), 3.76−3.80 (t, $J = 8.1$ Hz, 2H), 6.81 (s, 1H), 6.84−6.87 (m, 1H), 7.74 (d, $J = 8.5$ Hz, 1H), 8.93 (s, 1H). MS: m/z 147.9 (M + 1)$^+$.

Preparation of 1-(3-Methyl[1,2,4]oxadiazol-5-yl)propan-2-one (9a) (Scheme 4). To a solution of 2,2,6-trimethyl[1,3]-dioxin-4-one (15 g, 105 mmol) in dioxane (150 mL), **8** (8 g, 72.4 mmol) and triethylamine (8.8 g, 87 mmol) were added and refluxed for 15 h.[28] The mixture was cooled to room temperature, brine (100 mL) was added, and the emulsion was extracted with ethyl acetate (2 × 100 mL). NaOH (2 N, 50 mL) was added to the water layer and extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated in vacuo to give yellow crystals (**9a**) (4.8 g, 47%). ^{1}H NMR (DMSO-d_6): $\delta = 2.20$ (s, 3H), 2.28 (s, 3H), 4.29 (s, 2H). MS: m/z 140.9 (M + 1)$^+$.

Preparation of 5-Hydroxy-2-propyl-1H-indole-3-carboxylic Acid Ethyl Ester (11g) (Scheme 5). Ammonia was bubbled into a solution of **9g** in methanol (200 mL) at 5 °C for 15 min and then stirred at room temperature for 24 h to form **10g**. The reaction mixture was concentrated, and acetic acid (150 mL) and 1,4-benzoquinone (15.7 g, 0.145 mol) were added and stirred for 3 h at room temperature. The suspension was filtered, washed with CH$_2$Cl$_2$ (2 × 50 mL), and dried in a vacuum oven at 50 °C for 24 h to give a beige solid (**11g**) (2.2 g, 6%). ^{1}H NMR (DMSO-d_6): $\delta = 0.89$ (t, $J = 7.3$ Hz, 3H), 1.31 (t, $J = 7.1$ Hz, 3H), 1.65 (m, 2H), 2.96 (t, $J = 7.7$ Hz, 2H), 4.21 (q, $J = 7.1$ Hz, 2H), 6.57 (dd, $J = 8.4$ Hz, $^4J = 2.3$ Hz, 1H), 7.10 (d, $J = 8.4$ Hz, 1H), 7.30 (d, $^4J = 2.3$ Hz, 1H), 8.80 (s, 1H), 11.46 (s, 1H). MS: m/z 248.1 (M + 1)$^+$. The compounds **11a−f** and **11h−p** were obtained following the preparation similar to that of **11g**.

Preparation of 4-(Dimethylamino-methyl)-5-hydroxy-2-propyl-1H-indole-3-carboxylic Acid Ethyl Ester (12g) (Scheme 5). To a solution of **11g** (2.2 g, 8.9 mmol) in ethanol (20 mL) were added formaldehyde (37%, 0.85 mL, 10.7 mmol) and dimethylamine (40%, 2.2 mL, 19.6 mmol). The reaction mixture was stirred for approximately 16 h at 50 °C. The solution was then diluted with water (200 mL) and extracted

with CH$_2$Cl$_2$ (3 × 50 mL). The organic layer was dried (MgSO$_4$) and concentrated in vacuo. The dark oil was dissolved in ethanol (2 mL)/ether (10 mL) and HCl gas was bubbled into the solution for 1 min. This mixture was kept at 5 °C for approximately 16 h. The suspension was filtered, and the solid residue was basified with aqueous K$_2$CO$_3$ (10%), extracted with CH$_2$Cl$_2$ (3 × 50 mL), dried (Na$_2$SO$_4$), and concentrated in vacuo to give a brown solid (**12g**) (1.2 g, 44%). ^{1}H NMR (DMSO-d_6): $\delta = 0.87$ (t, $J = 7.3$ Hz, 3H), 1.27 (t, $J = 7.3$ Hz, 3H), 1.61 (m, 2H), 2.17 (s, 6H), 2.79−2.83 (m, 2H), 3.98 (s, 2H), 4.18 (q, $J = 7.3$ Hz, 2H), 6.55 (d, $J = 8.5$ Hz, 1H), 7.05 (d, $J = 8.5$ Hz, 1H), 11.40 (s, 1H). MS: m/z 305.1 (M + 1)$^+$. The compounds **12a−f** and **12h−s** were obtained following the preparation similar to that of **12g**.

Preparation of 2-Methyl-5-triisopropylsilanyloxy-1H-indole-3-carboxylic Acid Benzyl Ester (13) (Scheme 6). To a solution of **11d** (17.7 g, 62.9 mmol) and imidazole (10.7 g, 157 mmol) in dimethylformamide (DMF, 40 mL) was added chloro-triisopropyl-silane (14.55 g, 75.5 mmol). The reaction mixture was stirred for approximately 16 h, CH$_2$Cl$_2$ (150 mL) was added, and the solution was washed with brine (3 × 100 mL), dried (MgSO$_4$), concentrated in vacuo, and chromatographed (80% hexane, 20% ethyl acetate) to give a brown solid (**13**) (11.2 g, 41%). ^{1}H NMR (DMSO-d_6): $\delta = 0.93−0.97$ (m, 18H), 1.00−1.08 (m, 3H), 2.58 (s, 3H), 5.24 (s, 2H), 6.62−6.65 (m, 1H), 7.16 (d, $J = 8.5$ Hz, 1H), 7.30−7.43 (m, 6H), 11.71 (s, 1H). MS: m/z 438.3 (M + 1)$^+$.

Preparation of 2-Methyl-5-triisopropylsilanyloxy-1H-indole-3-carboxylic Acid (14) (Scheme 6). Compound **13** (11.2 g, 25.6 mmol) was dissolved in ethanol (200 mL) and debenzylated with 10% Pd/C (0,6 g) and H$_2$ in the presence of formic acid (88%, 10 mL). After 5 h, the solution was concentrated in vacuo to give **14** (8.8 g, 99%). ^{1}H NMR (DMSO-d_6): $\delta = 1.01−1.09$ (m, 21H), 2.55 (s, 3H), 6.61−6.64 (m, 1H), 7.13−7.15 (m, 1H), 7.40 (m, 1H), 11.53 (s, 1H), 11.70 (bs, 1H). MS: m/z 348.2 (M + 1)$^+$.

Preparation of 2-Methyl-5-triisopropylsilanyloxy-1H-indole-3-carboxylic Acid Ethyl-methyl-amide (11r) (Scheme 6). Compound **14** (8.8 g, 25.3 mmol) in DMF (50 mL) was added sequentially to PyBOP (17.7 g, 34 mmol), HOBT (4.6 g, 34 mmol), and tripropylamine (5 g, 35 mmol). The mixture was allowed to stir for approximately 16 h at room temperature. CH$_2$Cl$_2$ (200 mL) was added, and the organic layer was washed with brine (2 × 100 mL) and concentrated in vacuo. The residue was purified by chromatography (90% hexane, 10% ethyl acetate) and concentrated in vacuo to give an oil, which crystallized after standing at room temperature for 1 day (6.8 g, 58%). To a solution of residue (2.33 g, 5 mmol) in CH$_2$Cl$_2$ (20 mL), methyl-ethylamine (0.3 g, 5 mmol) was added and the reaction mixture was allowed to stir at room temperature for approximately 16 h. The organic layer was washed with brine (2 × 50 mL) and 2% HCl (2 × 50 mL), dried (MgSO$_4$), and concentrated in vacuo to give the desired product (**11r**) (1.6 g, 82%). ^{1}H NMR (DMSO-d_6): $\delta = 1.00−1.03$ (m, 21H), 1.16−1.21 (m, 3H), 2.30 (s, 3H), 2.88 (s, 3H), 3.37−3.38 (m, 2H), 6.59 (d, $J = 8.5$ Hz, 1H), 6.68 (s, 1H), 7.12 (d, $J = 8.5$ Hz, 1H), 11.15 (s, 1H). MS: m/z 389.2 (M + 1)$^+$.

Preparation of 2-Methyl-1H-indole-5-ol (15) (Scheme 6). A suspension of **11q** (7 g, 32 mmol) in aqueous HCl (20%, 500 mL) was refluxed for 2 h 30 min. The brown solution was cooled to room temperature and adjusted to pH 5−6 by adding aqueous 25% NaOH with cooling (5 °C). The resulting suspension was extracted with CH$_2$Cl$_2$ (3 × 150 mL), and the organic layer was dried (MgSO$_4$) and concentrated in vacuo to give a bright brown solid (**15**) (4 g, 85%). ^{1}H NMR (DMSO-d_6): $\delta = 2.27$ (s, 3H), 5.86 (s, 1H), 6.43 (dd, $J = 8.5$ Hz, $^4J = 2.2$ Hz, 1H), 6.66 (d, $J = 2.2$ Hz, 1H), 6.98 (d, $J = 8.5$ Hz, 1H), 8.43 (s, 1H), 10.49 (s, 1H). MS: m/z 147.9 (M + 1)$^+$.

Preparation of 3-Benzyl-2-methyl-1H-indol-5-ol (11s) (Scheme 6). To a solution of **15** (3.75 g, 25.6 mmol) in acetonitrile (100 mL) and K$_2$CO$_3$ (6.9 g, 50 mmol) was added benzylbromide (4.38 g, 25.6 mmol). The reaction mixture was refluxed for approximately 16 h, cooled to room temperature, and concentrated in vacuo. The residue was purified by

chromatography (80% hexane, 20% ethyl acetate) to give the desired product **11s** (1.4 g, 23%). MS: m/z 238.1 $(M + 1)^+$.

1. Binding Studies. Materials and Methods. Cell Growth Conditions. CHO-K1 cell lines expressing the five subtypes of muscarinic receptors were initially obtained from Dr. M. R. Brann (NINDS; University of Vermont) and grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% nonessential amino acids. The cells were passed on a weekly basis, and receptor levels appeared stable for up to four months in culture. The total number of receptors was controlled by the confluency of the cells with the highest number of receptors observed just below confluency. For binding studies, cells were harvested at 70–90% confluency using light trypsinization. To harvest membranes, cells were washed with phosphate-buffered saline (PBS), scraped into ice cold 5 mM Tris-HCl with ethylenediaminetetraacetic acid (EDTA), homogenized using a Polytron disrupter, sonicated, and then pelleted at 20 000 rpm for 30 min. Membranes were resuspended in 10 mM Na^+/K^+ PO_4 buffer, aliquoted, and kept frozen at −80 °C until the day of assay.

Cell Membrane Binding. Membrane aliquots of between 10 and 40 mg were added to 2 mL volumes of 10 mM Na^+/K^+ PO_4, pH 7.4, containing appropriate concentrations of test compound and 0.1 nM [^{3}H]NMS. Atropine at 1 mM was used to determine nonspecific binding. Incubation proceeded for 120 min at 25 °C with constant agitation and was terminated by rapid vacuum filtration through GF/B Whatman filters using a Brandel Cell Harvester. Filters were washed three times with 5 mL of ice-cold 10 mM Na^+/K^+ PO_4, placed in vials with 10 mL of Beckman Ready Gel, allowed to sit for approximately 16 h, vortexed, and then counted. IC_{50} values were obtained using a logit equation adapted from Parker and Waud,[29] with K_i values calculated using the equation of Cheng and Prusoff.[30]

2. Neurochemical Effects. Materials. 3-Hydroxybenzyl-hydrazine dihydrochloride (NSD 1015) was purchased from the Sigma Chemical Co., St. Louis, MO. The Department of Chemistry, Parke-Davis Pharmaceutical Research, synthesized CI-1017 and **24**. All other compounds used were purchased from commercial sources.

Animals. Male Long–Evans rats (l80–250 g) from Charles River Laboratories (Wilmington, MA) were used for all studies. Animals were maintained under a 12 h light–dark cycle in accordance with guidelines established by the National Institute of Health and American Association for the Accreditation of Laboratory Animal Care. Free access was provided to standard chow and water. Doses of test compounds were calculated as the free base and administered as suspension with 0.1% Tween 80.

Dopa Accumulation. Interactions with rat brain striatal and mesolimbic muscarinic receptors were examined by measuring DOPA accumulation after inhibiting L-aromatic amino acid decarboxylase activity with NSD 1015.[31] Test compounds were administered 30 min before NSD 1015 (100 mg/kg, ip) and sacrificed 30 min after the NSD 1015. The brain was quickly removed and placed on an ice-cooled plate for dissection of striatum and mesolimbic regions. The concentration of DOPA was determined by using high-pressure liquid chromatography with electrochemical detection as described previously in detail.[32]

Statistical Analysis. Statistical comparisons were made with the use of analysis of variance (ANOVA) followed by a Newman–Keuls test that was used to determine which group means were significantly different.

3. LMA. LMA testing in rats was performed using 16 Beam Digiscan Animal Activity Monitors (Accuscan Electronics, Columbus, OH). Each test chamber consisted of a Plexiglas box measuring 16 in × 16 in. Rats were tested one per chamber.

Male Sprague–Dawley rats (Harlan Labs), 180–200 g, were used. Rats were housed six per cage with ad lib access to food and water for 7–10 days after arrival. The housing room lights were set on a 12 h light:12 h dark cycle (lights on at 6 a.m.). Rats were food-deprived for 24 h before testing. On the test day, the rats were moved in their housing cages from the housing room to the testing room. Rats remained in gang cages in the test room during the predosing period. The rats were weighed and dosed in the test room, with the room lights off and dim illumination provided from a small desk lamp. The white noise generator was on. Thirty minutes after dosing, the rats were placed into the testing chambers and locomotor activity was recorded for 60 min. Following the last rat being placed into a chamber, the desk lamp was turned off. Dim light was provided by the presence of two computer monitors in the room. LMA is expressed as centimeters traveled. Statistical analysis was performed using a one way ANOVA followed by a posthoc Student–Newman–Keuls or Dunn's test. Vehicle or **24** was administered orally in a volume of 5 mL/kg. Compound **24** was suspended in 0.5% methocel in water.

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